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ORIGINAL PAPER

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Rhizobium etli maize populations and their competitiveness for root colonization

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Abstract *Rhizobium etli*, which normally forms nitrogen-fixing nodules on *Phaseolus vulgaris* (common bean), is a natural maize endophyte. The genetic diversity of *R. etli* strains from bulk soil, bean nodules, the maize rhizosphere, the maize root, and inside stem tissue in traditional fields where maize is intercropped with *P. vulgaris*-beans was analyzed. Based on plasmid profiles and alloenzymes, it was determined that several *R. etli* types were preferentially encountered as putative maize endophytes. Some of these strains from maize were more competitive maize-root colonizers than other *R. etli* strains from the rhizosphere or from bean nodules. The dominant and highly competitive strain Ch24-10 was the most tolerant to 6-methoxy-2-benzoxazolinone (MBOA), a maize antimicrobial compound that is inhibitory to some bacteria and fungi. The *R. tropici* strain CIAT899, successfully used as inoculant of *P. vulgaris*, was also found to be a competitive maize endophyte in inoculation experiments.

Introduction

The grass family is agriculturally important because humans rely on grasses, including rice, wheat and maize, for a major portion of their diet and that of domestic animals (Kellog 2001). Maize is the basis of human nutrition in Latin American countries and, like most cereals, it requires high N fertilization for optimal yield. It has been pointed out that it would be a striking achievement if cereals could benefit from biological nitrogen fixation in partnership with associated bacteria (James et al. 2000; Triplett 1996).

Mexico is both the origin of maize-based agriculture and the center of the genetic diversity of this crop. Mesoamerican pre-hispanic cultures developed using maize and bean as basic crops, and even now traditional farmers depend on them for food. Genetic erosion of maize diversity has occurred by growing improved varieties using modern technology that has displaced traditional agriculture. Original maize land-races have been preserved only where traditional agriculture is maintained. In traditional agriculture, maize and bean are cultivated together with bean plants climbing on maize as stalks (intercropping). The roots are intermixed, and this facilitates the "sharing" of soil microbes, including symbiotic bacteria. *Rhizobium etli* is the bacterium that most commonly forms nitrogen-fixing nodules on *Phaseolus vulgaris* bean (reviewed in Martínez-Romero 2003), and it is also naturally associated with maize, residing not only in the rhizosphere, but also inside maize roots and stems (Gutiérrez-Zamora and Martínez Romero 2001). Maize endophytes have been described as plant growth promoter rhizobacteria (PGPRs) but have not been found to fix nitrogen (Chelius and Triplett 2000), as is also the case in other non-legume associations with diazotrophs (Fuentes-Ramírez et al. 1999; Biswas et al. 2000a,b; James 2000; Giller and Merckx 2003). Nevertheless, acetylene reduction activity and N incorporation in maize have been reported (Von Bülow and Döbereiner 1975; Rennie 1980; Alexander and Zuberer 1989) and García de Salamone et al. (1996) found that *Azospirillum* spp. inoculation accounted for significant levels of N_2-fixation depending on the maize genotype.

In Senegal and Guinea, West Africa, *Bradyrhizobium* spp. have been found within the roots of wetland wild rice (Chaintreuil et al. 2000), and *Azorhizobium caulinodans* has been found in the roots of rice land-races (Engelhard et al. 2000) and in the rhizosphere in rice of paddy fields in Asia (Ladha et al. 1989). In Egypt, *Rhizobium leguminosarum* bv. trifolii has been found as a natural endophyte of rice, where it promotes plant growth (Yanni et al. 1997). In contrast, some *R. leguminosarum* bv. trifolii isolates from clover nodules, such as ANU843, inhibit rice development, while derivatives cured of different plasmids did not affect growth (Perrine et al. 2001), suggesting that

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there is plasmidic genetic information that interferes with rice interaction, but which is required for clover nodulation.

Inside plants, bacterial endophytes encounter new niches where adapted clones may be selected. Endophytes must have specific mechanisms to colonize internal plant tissues (Kovtunovych et al. 1999) and adequately use plant nutrients without causing harm to the plant; they must also be able to survive plant defense reactions. Maize-associated bacteria and endophytes appear to have mechanisms allowing them to avoid the toxicity of plant cyclic hydroxamates. Therefore, in the present study, we tested the resistance of *R. etli* strains to 6-methoxy-2-benzoxazolinone (MBOA), the principal natural decomposition product of 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3-one (DIMBOA), a cyclic hydroxamic acid from corn. MBOA is inhibitory to plant pathogenic fungi and bacteria (Corcuera et al. 1978; Glenn et al. 2001).

It has long been considered that the *Rhizobium* strains in nodules only represent a fraction of the soil population, which implies that there is a selection by the plant of the most adapted or most competitive strains (Bromfield et al. 1995). Indeed, differences among *R. leguminosarum* bv. trifolii soil and clover nodule isolates were identified by PCR amplification and sequences analysis of *nodD* genes. In this case, soil rhizobial diversity was assessed from DNA isolated directly from the soil (Zézé et al. 2001). The frequency of *Sinorhizobium meliloti* genotypes isolated directly from soil differed from those of alfalfa and sweet clover nodules (Bromfield et al. 1995). We decided to test the hypothesis that endophytic bacterial genotypes were selected from the total population. Plasmid and alloenzyme patterns of maize putative endophytes were compared with those of *R. etli* isolates from the soil, rhizosphere, and bean nodules. To test whether maize putative endophytes were better adapted for maize colonization, intra-strain competition experiments were carried out.

Materials and methods

Strain isolation. Three-month-old maize plants were obtained from two sites: Cholula, and San Miguel Acuexcomac, Puebla, Mexico. Cultivars "Criollo Cholula" and "Criollo San Miguel Acuexcomac" were subsequently used. All isolates were from groups of five plants per field from traditional fields where maize is intercropped with *P. vulgaris*-bean. The Cholula site has a fertile soil with a sandy clay loam texture, pH 6.2, and a rainfall of 800–1,000 mm. The San Miguel Acuexcomac site has a less fertile soil with clay texture, pH 8.3, and is in a semi-arid location that receives a rainfall of 500–600 mm.

Rhizospheric isolates from maize roots were obtained from root-adhering soil by sonication, which releases adhered soil particles, as described (Streit et al. 1996). Bulk soil and rhizospheric samples were suspended in sterile water and aliquots of serial dilutions were inoculated on bean plants (Gutiérrez-Zamora and Martínez-Romero 2001). Inside root bacteria were isolated from the sonicated roots, which were subsequently sterilized by treating them with 70% ethanol for 1 min, followed by 1.2% sodium hypochlorite for 15 min, rinsing four times with sterilize water, once with 2% sodium thiosulfate, and then a final rinse of water before macerating the roots. Thiosulfate removes residual chlorine that may affect growth or induce mutagenesis (Miché and Balandreau 2001). Inside stem bacteria were isolated from sonicated stems, sterilized with 1.2% sodium hypochlorite for 10 min, rinsed with water and sodium thiosulfate as was carried out with the roots, and finally flamed with alcohol (except for 30-day-old plants). The outer layers of the stem were eliminated and inner tissues were macerated with sterilized 10 mM $MgSO_4$ to isolate the endophytic bacteria. We confirmed that roots and stems were superficially sterilized by placing them on PY (per liter: 5 g peptone, 3 g yeast extract, 1 g $CaCl_2 \cdot 2H_2O$) plates and incubating at 30°C for 3 days. Bean was used as a trap plant to select *R. etli* as described previously (Gutiérrez-Zamora and Martínez-Romero 2001).

R. etli quantification. CFUs were determined directly from the inoculated plants, grown in covered pots in the greenhouse, by plating serial dilutions of maize macerates or bacteria recovered from the maize rhizosphere on PY medium amended with cycloheximide (10 mg l^{-1}) and nalidixic acid (20 mg l^{-1}). *R. etli* strains are naturally nalidixic acid resistant. The identity of recovered bacteria was determined according to resistance to antibiotics and also by comparing the plasmid profiles of a few colonies with those of the inoculated strains. Since maize seeds naturally contain endophytic bacteria (Mc Inroy and Kloepper 1995), we sometimes found a few nalidixic-acid-resistant bacteria, even in the non-inoculated controls, but the colonies of these bacteria were morphologically distinguishable from *R. etli*. Isolates were tested for growth in LB medium (per liter: 10 g peptone, 5 g yeast extract, 10 g NaCl) and all bacteria growing in LB were discarded since *R. etli* does not grow in this medium.

Plasmid profiles. Modified Eckhardt gels (Hynes and McGregor 1990) were used to visualize plasmids. Plasmids were hybridized using ^{32}P-labeled PCR-synthesized probes (Wang et al. 1999) of the following *R. etli* CFN42 genes: *lpsβ* from plasmid b (García-de los Santos and Brom 1997), *rmrR* from plasmid b (González-Pasayo and Martínez-Romero 2000), *nifH* from plasmid d (Quinto et al. 1982), *cyaA*, from plasmid e (Tellez-Sosa, personal communication), and *fixL* from plasmid f (Girard et al. 2000).

Alloenzyme analysis. Extracts of bacterial isolates from maize putative endophytes and rhizospheric as well as bean nodule isolates were prepared from cultures grown in PY medium. Enzymatic activities for xanthine dehydrogenase, malate dehydrogenase, indophenol oxydase, glucose-6-phosphate dehydrogenase, isocitrate dehydrogenase, phosphoglucomutase, hexokinase, and alanine dehydrogenase were detected as described (Selander et al. 1986). Genetic distance between each pair of electrophoretic types (ETs) was estimated as the proportion of loci at which dissimilar alleles occurred, and cluster analysis was performed by the

Table 1 *Rhizobium* strains used in this study

Strain	Relevant characteristics	Plasmid pattern	Reference	Origin	Date of isolation
CIAT899	*R. tropici* type B, wild type		(Graham et al. 1982)		
Ch24-10	Maize stem isolate	A-1	This study	Cholula	August, 2000
Ch24-10 Sm^r	Ch 24-10 Sm^r mutant	A-1	This study	Cholula	August, 2000
Ch2-12	Bean nodule isolate	B-1	This study	Cholula	August, 2000
Ch4-22	Maize rhizosphere isolate	C-2	This study	Cholula	August, 2000
Ch2-15	Bean nodule isolate	C-3	This study	Cholula	August, 2000
SM12-7	Maize stem isolate	S-1	This study	San Miguel Acuexcomac	September, 2001
SM12-7 Sm^r	SM 12-7 Sm^r mutant	S-1	This study	San Miguel Acuexcomac	September, 2001
SM44-1	Maize rhizosphere isolate	U-1	This study	San Miguel Acuexcomac	September, 2001

average-linkage method from a matrix of pairwise genetic distance (Selander et al. 1986).

Competition experiments. Maize seeds from cultivar "Criollo de Amatlán" were sterilized as described above for roots, but by shaking them. Seeds were germinated in 0.75% water-agar in petri dishes for 48 h and then transplanted to 1-l pots (two plants per pot) with vermiculite that had been sterilized in an autoclave for 2 h. Sterility was tested by placing a sample of vermiculite on PY plates and incubating at 30°C for 5 days. Maize seeds were inoculated with 10^7 cells per plant, distributing the bacteria in Fahraeus medium (Fahraeus 1957). Plants were grown in pots by covering the surface with aluminum foil and using sterilized cotton to cover the opening around each plant stem. Pots were maintained in the greenhouse and sampled at 1 month. Acetylene reduction activity was measured as described (Gutiérrez-Zamora and Martínez-Romero 2001).Streptomycin-resistant (Sm^r 100 mg l^{-1}) mutants were obtained for the maize stem-borne strains (Ch24-10 from Cholula and SM12-7 from San Miguel Acuexcomac). Spontaneous mutants were selected by plating the wild-type strains on PY medium containing 100 mg Sm l^{-1}. Afterwards, they were inoculated onto maize plants in a 1:1 ratio with the wild-type strains to test whether the antibiotic-resistant mutants were as competitive as the original strains in colonizing the rhizosphere and the root. The reference strains selected to be inoculated together with the maize stem-borne strains Ch24-10 and SM12-7 were: SM44-1 and Ch4-22 from maize rhizosphere, Ch2-12 and Ch2-15 from bean nodules, and *R. tropici* CIAT899. The *Rhizobium* strains used and their combinations are listed in Tables 1 and 4. The inoculum ratios were estimated by OD, and by plating serial dilutions. The outcome of strain proportions was obtained by serial dilutions in selective media, and more than 200 CFU were screened from each plant. These were compared to the actual ratio inoculated and then analyzed using Student's *t* test taking this ratio into account.

MBOA resistance analysis. MBOA (Sigma) was dissolved in ethanol and tested at 0.1, 0.25, 0.50, and 0.75 g l^{-1} in liquid PY medium. Bacterial growth was determined spectrophotometrically at 600 nm every 4 h for the first 12 h and then at 24 and 48 h.

Results

Plasmid patterns were characterized by genetic markers. Plasmid patterns of the isolated strains (Fig. 1) were determined by hybridization to markers known to be plasmid located in *R. etli* CFN42. Although this was mainly done to help group the plasmid profiles, it is worth noting the following: we observed that the genes *lpsβ* and *rmrR* were always found on the same plasmid (Fig. 2) and they

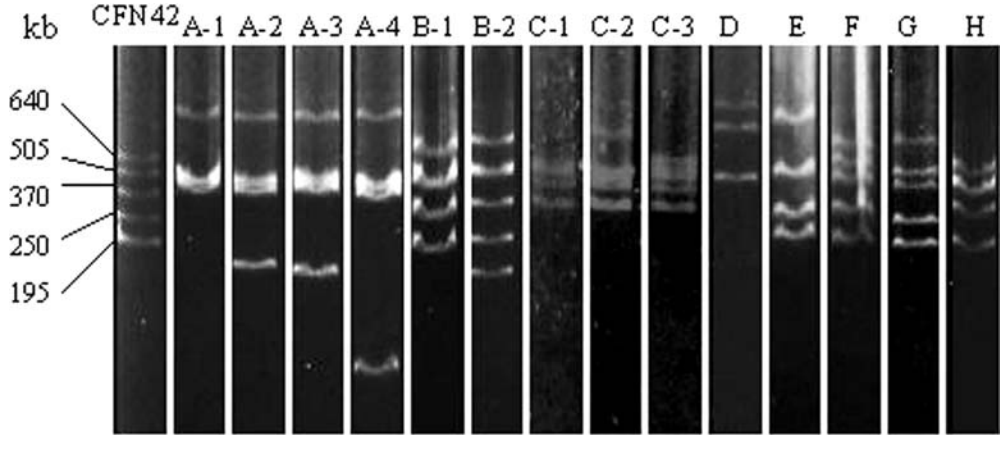

Fig. 1 Eckhardt gel showing the plasmid profiles found in isolates from Cholula

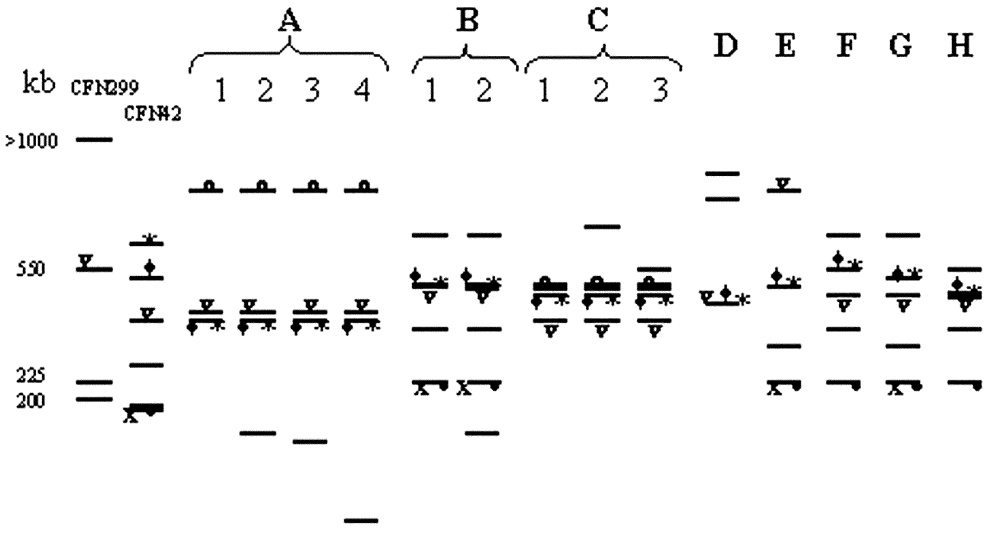

Fig. 2 Plasmid profiles found in isolates from Cholula, showing the position of plasmids hybridizing with *nifH* (▽), *cyaA* (♦), *fixL* (*), *lpsβ* (•), *lpsβ* (with faint hybridizing signal) (○), and *rmrR* (×)

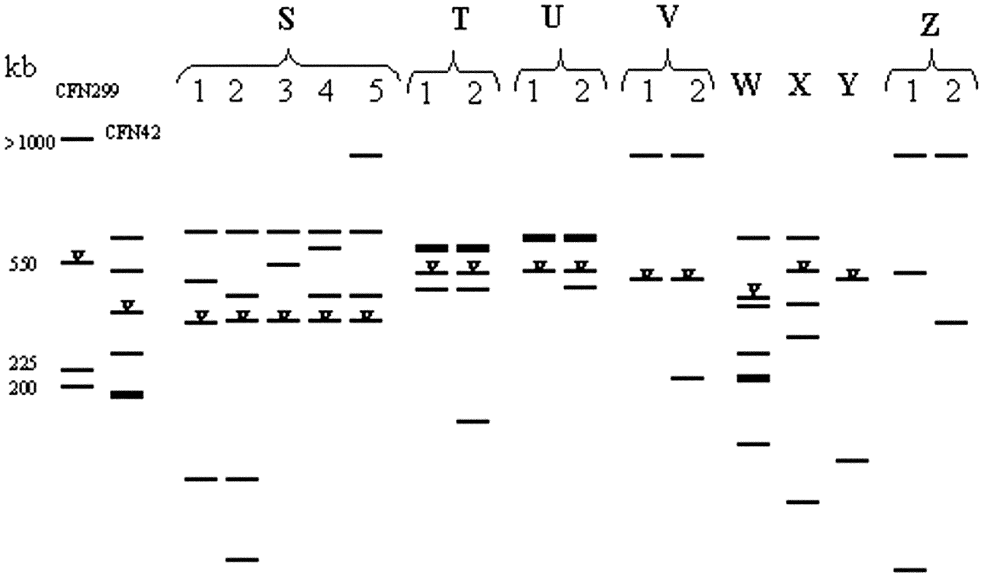

Fig. 3 Plasmid profiles found in isolates from San Miguel Acuexcomac, showing the position of plasmids hybridizing with *nifH* (▽)

have been previously reported to be linked in CFN42 (González-Pasayo and Martínez-Romero 2000). Also, *cyaA* and *fixL* were usually on the same plasmid, except in strain CFN42. Similar to Brom et al. (2002), we found that *nifH*, *fixL*, and *lpsβ* were frequently located on three distinct replicons, except for pattern D, which has *nifH* and *fixL* on the same plasmid. Most of the *R. etli* strains tested were found to carry a plasmid containing a *fixL* homolog (Brom et al. 2002). In contrast, we found that not all *R. etli* isolates hybridized strongly to *lpsβ*, which may indicate a high divergence in this locus.

Maize stem R. etli isolates are of limited genotypes. Plasmid content was analyzed in 191 *Rhizobium* isolates from Cholula and 72 isolates from San Miguel Acuexcomac. In the case of San Miguel Acuexcomac isolates, the plasmids were only hybridized with *nifH*. In several cases, almost identical plasmid patterns were found; these variants were designated by numbers and a letter (Figs. 1, 2, 3). The relative frequency of the various plasmid patterns was expressed as % of isolates with a single plasmid profile referred to the total *R. etli* isolates from a particular condition (stem or root putative endophytes, rhizosphere, bean nodules, or soil). The relative frequency of the plasmid patterns differed in isolates obtained from inside stem and root, rhizosphere, bean nodules, and soil (Tables 2, 3). The bacteria recovered from inside maize stems all had a single plasmid pattern (100% relative frequency) (A-1 in Cholula and S-1 in San Miguel Acuexcomac). The isolates recovered from inside roots had more plasmid types than the stem isolates. The most abundant types inside maize roots were A-1 and G in Cholula (relative frequency of 43.7 and 33.3%, respectively) and S-2 and U-1 in San Miguel Acuexcomac (relative frequency of 64.3 and 21.5%, respectively). Most of the different plasmid patterns were represented in the isolates obtained from the rhizosphere, although some types dominated, such as A-1 in Cholula and U-1 in San Miguel Acuexcomac. In addi-

Table 2 Relative frequency (%) of different plasmid profiles in a traditionally cultivated field of intercropped maize and bean (Cholula, Puebla, Mexico)

Plasmid profile	Maize stem	Maize root	Maize rhizosphere	Bean nodules	Soil
A-1	100	43.7	34.1	28.5	5.4
A-2			9.8	25.0	
A-3			4.9		5.4
A-4		6.3	7.3	10.7	
B-1			19.5	14.3	46.0
B-2			4.9		21.6
C-1				3.6	
C-2			2.4		
C-3			4.9	10.7	
D		8.3			
E					2.7
F		6.3	4.9		2.7
G		33.3		3.6	
H		2.1	7.3	3.6	16.2
Total strains analyzed	37	48	41	28	28

Table 3 Relative frequency (%) of different plasmid profiles in a traditionally cultivated field of intercropped maize and bean (San Miguel Acuexcomac, Mexico)

Plasmid profile	Maize stem	Maize root	Maize rhizosphere	Bean nodules	Soil
S-1	100		5.0		6.2
S-2		64.3			
S-3			10.0	9.1	
S-4			5.0		12.5
S-5			10.0		
T-1		7.1		18.2	6.2
T-2				9.1	
U-1		21.5	45.0	18.2	31.3
U-2		7.1	15.0	9.1	6.2
V-1			5.0	18.2	
V-2					6.2
W					12.5
X				9.1	
Y			5.0		
Z-1					12.5
Z-2				9.1	6.2
Total strains analyzed	11	14	20	11	16

tion, some plasmid types were only found in nodule isolates.

Twenty-seven strains from Cholula and nine from San Miguel Acuexcomac, representing different plasmid profiles, were analyzed based on their metabolic enzyme patterns. Figure 4 shows that all stem endophytes fall into two ET: one of the seven ETs encountered in Cholula, and one of the two ETs encountered in San Miguel Acuexcomac. We observed that the ETs were related to the plasmid patterns. Strains with plasmid patterns A or C were clustered in two ETs, which were separated at a genetic distance of only 0.12. Strains with plasmid pattern B were grouped in three ETs with a genetic distance of 0.17 between them. The ones from plasmid pattern G and H grouped with the B type. This was also observed in San Miguel Acuexcomac, as strains with plasmid pattern S were in a different ET than the strains with plasmid patterns U or W.

The dendogram (Fig. 4) shows that most of the isolates were related to *R. etli* reference strain CFN42. It remains to be established whether cluster II also corresponds to *R. etli*.

Some maize-stem R. etli strains are highly competitive. To check whether a strain belonging to the dominant plasmid pattern inside the stem was more competitive in its ability to penetrate the plant than other rhizosphere or bean nodule isolates, competition experiments were carried out (Table 4). The maize stem-borne strain from Cholula Ch24-10 Sm^r was more competitive than Ch4-22 (a rare maize rhizosphere isolate) or Ch2-15 (a bean nodule isolate). Strain Ch24-10 Sm^r was better able to colonize not only maize root but also maize rhizosphere. This correlated with its success as a maize rhizospheric bacteria in the fields since the A-1 pattern was found also at a higher frequency in the rhizosphere and in roots of maize than in the soil (Table 2). This was not always the case, since the isolate with plasmid pattern G in Cholula, an abundant pattern inside the root, was not detected in the maize rhizosphere.

The soil- and rhizosphere-dominant plasmid types B-1 of Cholula (represented by strain Ch2-12), and U-1 of San Miguel Acuexcomac (represented by strain SM44-1) were not the most frequent types isolated from the maize stem (Tables 2, 3). However, strains with these plasmid patterns inoculated in competition with strains carrying plasmid types from the stem were just as competitive as the maize-borne putative endophyte (Table 4).

All the strains tested in the competition assays were screened for their resistance to MBOA. Ch24-10 was the only strain resistant to 0.75 g l^{-1}. At this concentration growth of all the other strains was completely inhibited whereas at 0.10 g l^{-1} only the growth of less competitive strains (Ch2-15 and Ch4-22) was inhibited (data not shown).

R. tropici CIAT899 was as competitive as Ch24-10 Sm^r (Table 4). Furthermore, plant heights were significantly different ($p<0.05$, Student's t test) when maize was inoculated with CIAT899. The increase in height was of 50%, similar to that of maize plants inoculated with *R. etli* (Gutiérrez-Zamora and Martínez-Romero 2001).

In maize inoculation experiments, rhizospheric bacteria were more numerous (10^6–10^8 CFU/g of dry soil) than root endophytic bacteria (10^3–10^4 CFU/g of fresh tissue). Acetylene was not reduced in any of the plants inoculated with either *R. etli* or *R. tropici* strains in spite of the large amount of rhizobia in the plants (data not shown). No *R. etli* isolates were recovered in non-inoculated maize plants.

Discussion

Maize is one of the most widely cultivated cereals in the world and its production is highly dependent on chemical

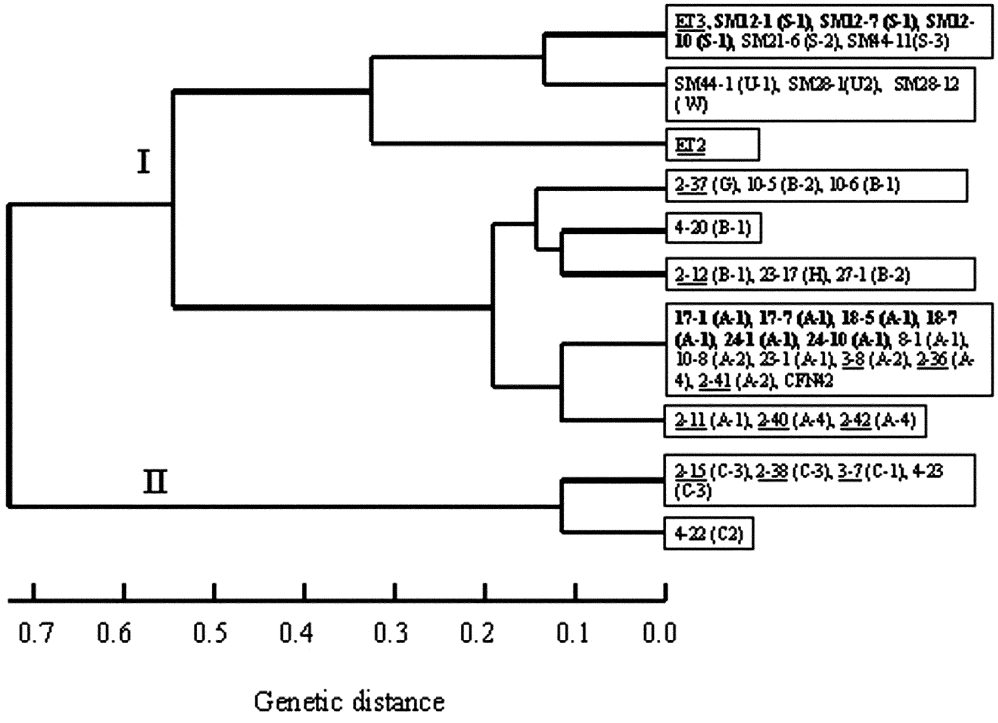

Fig. 4 Dendogram showing the genetic relatedness among alloenzyme patterns and plasmidic genotypes of *Rhizobium* strains isolated from San Miguel Acuexcomac (SM), and Cholula. ET2 and ET3 are strains isolated from the same field of San Miguel Acuexcomac in previous years (Silva et al. 2002). Maize stem endophyte strains are in *bold* face, bean isolates are *underlined*, and plasmid types are shown in *parenthesis*

Table 4 Intra-strain competition of maize stem-borne strains and reference strains for occupancy of the rhizosphere and the root of maize

Strains in inoculum		Ratio inoculated	Occupancy (%)			
			Rhizosphere		Root	
Reference strain	Maize stem strain		Reference strain	Maize stem strain	Reference strain	Maize stem strain
Ch2-12	Ch24-10 Sm^r	1.3:1	44.0	56.0	43.8	56.2
Ch4-22	Ch24-10 Sm^r	0.5:1	10.4	89.6[a]	7.8	92.2[a]
Ch2-15	Ch24-10 Sm^r	1.3:1	3.2	96.8[a]	8.5	91.5[a]
SM44-1	SM12-7 Sm^r	1:1	50.6	49.4	60.0	40.0
CIAT899	Ch24-10 Sm^r	1.3:1	50.0	50.0	73.3	26.7

[a]The values are significantly different ($p \leq 0.01$) with Student's t test, considering the initial ratio inoculated

fertilizers. Thus, it would be a significant achievement if maize could benefit from biological nitrogen fixation (Triplett 1996; Dobereiner et al. 1995). Ongoing research efforts on the subject are driven by this interest, and a diversity of diazotrophs have been identified from maize (Chelius and Triplett 2000, 2001); however, with exceptions (García de Salamone et al. 1996), nitrogen fixation in maize has not been clearly documented (Chelius and Triplett 2000; Gutiérrez-Zamora and Martínez-Romero 2001). In this work, acetylene reduction activity was not detected in spite of the large numbers of *R. etli* found to be associated with maize.

Diluting the inocula allowed the selection of uncommon rhizobia that may not be able to nodulate in the presence of more competitive isolates (Bala et al. 2001). For this reason, we most likely obtained an adequate representation of the soil, rhizospheric, and maize putative endophytic bacteria with our approach using bean as a plant trap. Inside bean nodules, non-nodulating bacteria were not encountered as contaminants. *R. tropici* CIAT899 was as competitive as strain Ch24-10 Sm^r in maize, implying that it may be possible to find *R. tropici* inside maize. However, up to now we have not encountered *R. tropici* strains as a natural maize endophyte in Mexican fields. Furthermore, in Mexico, *R. tropici* has not even been found in bean nodules, but has been reported as nodulating *Gliricidia sepium* (Acosta-Durán and Martínez-Romero 2002). *R. tropici* is an outstanding inoculant for *P. vulgaris* beans in South America (reviewed in Hungria et al. 2000) but it is not competitive for nodulating bean in the presence of *R. etli* (Martínez-Romero and Rosenblueth 1990).

Plasmid profile analysis is considered a good method when many rhizobia isolates are to be typed (Hartmann and Amarger 1991). Plasmid patterns were used to reveal a larger diversity of rhizobial endophytes in rice in flooded soils than in rice in drained soils (Yanni et al. 2001). Plasmid patterns correlated with DNA-fingerprint, alloenzyme, and BOX-PCR analyses (Hartmann and Amarger 1991; Silva et al. 2002; Yanni et al. 2001) and with the enzyme ETs presented here. It has been previously found that the plant host imposes strong selective pressure, which favors the maintenance of particular chromosome–plas-

mid associations (Silva et al. 2002). Our results showing different frequencies of *R. etli* genotypes in stems and soil resemble those obtained from insertion sequence hybridization analysis of isolates from soil and nodules of *Medicago sativa*, in which the frequency of *S. meliloti* genotypes was different in soil and nodules (Bromfield et al. 1995). The presence of dominant plasmid and alloenzyme ETs in stems suggests the existence of genotypes better adapted to internally colonize maize. An alternative explanation is that the endophytes are only a subset of the rhizospheric or soil population without any specific selection by the plant. We previously recovered from maize roots larger numbers of maize-borne strains and lower numbers of bacteria from our bean nodule collection, in single strain inoculation assays (Gutiérrez-Zamora and Martínez-Romero 2001). Thus we considered it of interest to test whether maize endophytic isolates were more competitive than rhizospheric or bean nodule strains for maize colonization. This was indeed observed in some cases, such as with Ch24-10 (strain with plasmid profile A-1 isolated from stems), which was better able to colonize both the roots and the rhizosphere. Successful colonization of the maize rhizosphere seemed to be related to successful endophytic colonization of the root. This may explain why some dominant maize rhizosphere plasmid types (B-1 of Cholula and U-1 of San Miguel Acuexcomac) were highly competitive for internal root maize colonization, even though they were not found inside maize stems (from field isolates). This result suggests that, in the field, other competing microorganisms affect colonization of the dominant rhizospheric strains, or that our stem sample was too small as to detect these strains.

By identifying a highly competitive strain for maize colonization the genetic determinants involved in this process can be studied. One of these genetic determinants may be related to resistance to MBOA and could confer a selective advantage to Ch24-10 for rhizosphere, root, and stem colonization in the field. This will be tested by analyzing the genes involved, their presence in other maize *R. etli* endophytes, and their role in colonization. To our knowledge, this is the first report of in vitro competition experiments using endophytes, which seem to be very useful in detecting better adapted strains, as has been observed previously in inter-strain competition assays for legume nodulation (Rosenblueth et al. 1998). While some candidates were not tested, such as isolates from group G that are found frequently inside the root, the population analysis carried out in the present study allowed the identification of highly competitive strains that may have agricultural applications.

It is worth mentioning that more morphologically diverse bacteria were reproducibly recovered from the non-inoculated controls than from *R. etli* inoculated maize plants, suggesting that *R. etli* displaces other maize endophytes and therefore seems to be more competitive. If this is so, *R etli* strains could be evaluated as inoculants of maize plants in Europe, which normally harbor *Burkholderia cepacia* strains that are potentially dangerous for humans (Balandreau et al. 2001; Chiarini et al. 2000).

Similar levels of endophytic bacterial populations have been obtained with *R. leguminosarum* bv. trifolii in rice (10^6–10^8 CFU/g fresh tissue) (Yanni et al. 1997). We have always been able to isolate inoculated *R. etli* strains from maize plants up to 3 months after inoculation, in contrast to what has been observed in *Azoarcus* endophytes of Kallar grass (Hurek et al. 2002) and in *Rhizobium* sp. endophytes of rice (Biswas et al. 2000b), where the endophytes become unculturable a few months after inoculation.

After inoculation, most of the bacteria remain in the rhizosphere, thus it is reasonable to assume that these vastly greater numbers of bacteria are much more likely to exert an effect on the inoculated plants than the considerably lower numbers in the root interior. As there is only one report that has addressed the role of endophytes vs rhizospheric bacteria (Gyaneshwar et al. 2002), further studies are needed

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References

Acosta-Durán C, Martínez Romero E (2002) Diversity of rhizobia from nodules of the leguminous tree *Gliricidia sepium*, a natural host of *Rhizobium tropici*. Arch Microbiol 178:161–164

Alexander DB, Zuberer DA (1989) $^{15}N_2$ fixation by bacteria associated with maize roots at a low partial O_2 pressure. Appl Environ Microbiol 55:1748–1753

Bala A, Murphy P, Giller KE (2001) Genetic diversity of rhizobia from natural populations varies with the soil dilution sampled. Soil Biol Biochem 33:841–843

Balandreau J, Viallard V, Cournoyer B, Coenye T, Laevens S, Vandamme P (2001) *Burholderia cepacia* genomovar III is a common plant-associated bacterium. Appl Environ Microbiol 67:982–985

Biswas JC, Ladha JK, Dazzo FB (2000a) Rhizobia inoculation improves nutrient uptake and growth of lowland rice. Soil Sci Soc Am J 64:1644–1650

Biswas JC, Ladha JK, Dazzo FB, Yanni YG, Rolfe BG (2000b) Rhizobial inoculation influences seedling vigor and yield of rice. Agron J 92:880–886

Brom S, Girard L, García-de los Santos A, Sanjuan-Pinilla JM, Olivares J, Sanjuán J (2002) Conservation of plasmid-encoded traits among bean-nodulating *Rhizobium* species. Appl Environ Microbiol 68:2555–2561

Bromfield ESP, Barran LR, Wheatcroft R (1995) Relative genetic structure of a population of *Rhizobium meliloti* isolated directly from soil and from nodules of alfalfa (*Medicago sativa*) and sweet clover (*Melilotus alba*). Mol Ecol 4:183–188

Chaintreuil C, Giraud E, Prin Y, Lorquin J, Bâ A, Gillis M, De Lajudie P, Dreyfus B (2000) Photosynthetic bradyrhizobia are natural endophytes of the African wild rice *Oryza breviligulata*. Appl Environ Microbiol 66:5437–5447

Chelius MK, Triplett EW (2000) Immunolocalization of dinitrogenase reductase produced by *Klebsiella pneumoniae* in association with *Zea mays* L. Appl Environ Microbiol 66:783–787

Chelius MK, Triplett EW (2001) The diversity of Archaea and Bacteria in association with the roots of *Zea mays* L. Microb Ecol 41:252–263

Chiarini L, Giovannelli V, Bevivino A, Dalmastri C, Tabacchioni S (2000) Different portions of the maize root system host *Burkholderia cepacia* populations with different degrees of genetic polymorphism. Environ Microbiol 2:111–118

Corcuera LJ, Woodward MD, Helgeson JP, Kelman A, Upper CD (1978) 2,4-Dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one, an inhibitor from *Zea mays* with differential activity against soft rotting *Erwinia* species. Plant Physiol 61:791–795

Dobereiner J, Urquiaga S, Boddey RM (1995) Alternatives for nitrogen nutrition of crops in tropical agriculture. Fert Res 42: 339–346

Engelhard M, Hurek T, Reinhold-Hurek B (2000) Preferential occurrence of diazotrophic endophytes, *Azoarcus* spp., in wild rice species and land races of *Oryza sativa* in comparison with modern races. Environ Microbiol 2:131–141

Fahraeus G (1957) The infection of clover root hair by nodule bacteria studied by a single glass slide technique. J Gen Microbiol 16:374–381

Fuentes-Ramírez LE, Caballero-Mellado J, Sepúlveda J, Martínez-Romero E (1999) Colonization of sugarcane by *Acetobacter diazotrophicus* is inhibited by high N-fertilization. FEMS Microbiol Ecol 29:117–128

García-de los Santos A, Brom S (1997) Characterization of two plasmid-borne *lpsβ* loci of *Rhizobium etli* required for lipopolysaccharide synthesis and for optimal interaction with plants. Mol Plant Microbe Interact 10:891–902

García de Salamone IEG, Dobereiner J, Urquiaga S, Boddey RM (1996) Biological nitrogen fixation in *Azospirillum* strain-maize genotype associations as evaluated by the superior 1 superior 5N isotope dilution technique. Biol Fertil Soils 23:249–256

Giller KE, Merckx R (2003) Exploring the boundaries of N_2-fixation in cereals and grasses: an hypothetical and experimental framework. Symbiosis 35:3–17

Girard L, Brom S, Dávalos A, López O, Soberón M, Romero D (2000) Differential regulation of *fixN*-reiterated genes in *Rhizobium etli* by a novel *fixL–fixK* cascade. Mol Plant Microbe Interact 13:1283–1292

Glenn AE, Hinton DM, Yates IE, Bacon CW (2001) Detoxification of corn antimicrobial compounds as the basis for isolating *Fusarium verticillioides* and some other *Fusarium* species from corn. Appl Environ Microbiol 67:2973–2981

González-Pasayo R, Martínez-Romero E (2000) Multiresistance genes of *Rhizobium etli* CFN42. Mol Plant Microbe Interact 13: 572–577

Graham PH, Viteri SE, Mackie F, Vargas AAT, Palacios A (1982) Variation in acid soil tolerance among strains of *Rhizobium phaseoli*. Field Crops Res 5:121–128

Gutiérrez-Zamora ML, Martínez-Romero E (2001) Natural endophytic association between *Rhizobium etli* and maize (*Zea mays* L.). J Biotechnol 91:117–126

Gyaneshwar P, James EK, Reddy PM, Ladha JK (2002) *Herbaspirillum* colonization increases growth and nitrogen accumulation in aluminium-tolerant rice varieties. New Phytol 154: 131–145

Hartmann A, Amarger N (1991) Genotypic diversity of an indigenous *Rhizobium meliloti* field population assessed by plasmid profiles, DNA fingerprinting, and insertion sequence typing. Can J Microbiol 37:600–608

Hungria M, Vargas MAT, Campo RJ, Chueire LMO, De S Andrade D (2000) The Brazilian experience with the soybean (*Glycine max*) and common bean (*Phaseolus vulgaris*) symbioses. In: Pedrosa FO, Hungria M, Yates G, Newton WE (eds) Nitrogen fixation: from molecules to crop productivity. Kluwer, Dordrecht, The Netherlands, pp 515

Hurek T, Handley LL, Reinhold-Hurek B, Piché Y (2002) *Azoarcus* grass endophytes contribute fixed nitrogen to the plant in an unculturable state. Mol Plant Microbe Interact 15:233–242

Hynes MF, McGregor NF (1990) Two plasmids other than the nodulation plasmid are necessary for formation of nitrogen-fixing nodules by *Rhizobium leguminosarum*. Mol Microbiol 4: 567–574

James EK (2000) Nitrogen fixation in endophytic and associative symbiosis. Field Crops Res 65:197–209

James EK, Gyaneshwar P, Barraquio WL, Mathan N, Ladha JK (2000) Endophytic diazotrophs associated with rice. In: Ladha JK, Reddy PM (eds) The quest for nitrogen fixation in rice. IRRI, Makati City, Philippines, pp 119–140

Kellog EA (2001) Evolutionary history of the grasses. Plant Physiol 125:1198–1205

Kovtunovych G, Lar O, Kamalova S, Kordyum V, Kleiner D, Kozyrovska N (1999) Correlation between pectate lyase activity and ability of diazotrophic *Klebsiella oxytoca* VN 13 to penetrate into plant tissues. Plant Soil 215:1–6

Ladha JK, García M, Miyan S, Padre A, Watanabe I (1989) Survival of *Azorhizobium caulinodans* in the soils and rhizosphere of wetland rice under *Sesbania rostrata*-rice rotation. Appl Environ Microbiol 55:454–460

Martínez-Romero E (2003) Diversity of *Rhizobium-Phaseolus vulgaris* symbiosis: overview and perspectives. Plant Soil 252:11–23

Martínez-Romero E, Rosenblueth M (1990) Increased bean *Phaseolus vulgaris* L. nodulation competitiveness of genetically modified strains. Appl Environ Microbiol 56:2384–2388

Mc Inroy JA, Kloepper JW (1995) Survey of indigenous bacterial endophytes from cotton and sweet corn. Plant Soil 173:337–342

Miché L, Balandreau J (2001) Effects of rice seed surface sterilization with hypochlorite on inoculated *Burkholderia vietnamiensis*. Appl Environ Microbiol 67:3046–3052

Perrine FM, Prayitno J, Weinman JJ, Dazzo FB, Rolfe BG (2001) *Rhizobium* plasmids are involved in the inhibition or stimulation of rice growth and development. Aust J Plant Physiol 28:923–937

Quinto C, de la Vega H, Flores M, Fernández L, Ballado T, Soberón G, Palacios R (1982) Reiteration of nitrogen fixation gene sequences in *Rhizobium phaseoli*. Nature 299:724–728

Rennie RJ (1980) ^{15}N-isotope dilution as a measure of dinitrogen fixation by *Azospirillum brasilense* associated with maize. Can J Bot 58:21–24

Rosenblueth M, Hynes MF, Martínez-Romero E (1998) *Rhizobium tropici teu* genes involved in specific uptake of *Phaseolus vulgaris* bean-exudate compounds. Mol Gen Genet 258:587–598

Selander RK, Caugant DA, Ochman H, Musser JM, Gilmour MN, Whittam TS (1986) Methods of multilocus enzyme electrophoresis for bacterial population genetics and systematics. Appl Environ Microbiol 51:873–884

Silva C, Vinuesa P, Eguiarte LE, Martínez-Romero E, Souza V (2002) *Rhizobium etli* and *Rhizobium gallicum* nodulate common bean in a traditionally managed milpa plot in Mexico: population genetics and biogeographic implications. Appl Environ Microbiol 69:884–893

Streit WR, Joseph CM, Phillips DA (1996) Biotin and other water-soluble vitamins are key growth factors for alfalfa rhizosphere colonization by *Rhizobium meliloti* 1021. Mol Plant Microbe Interact 5:330–338

Triplett EW (1996) Diazotrophic endophytes: progress and prospects for nitrogen fixation in monocots. Plant Soil 186:29–38

Von Bülow CFW, Döbereiner J (1975) Potential for nitrogen fixation in maize genotypes in Brazil. Proc Natl Acad Sci USA 72:2389–2393

Wang ET, Rogel MA, García-de los Santos A, Martínez-Romero J, Cevallos MA, Martínez-Romero E (1999) *Rhizobium etli* bv. mimosae, a novel biovar isolated from *Mimosa affinis*. Int J Syst Bacteriol 49:1479–1491

Yanni YG, Rizk RY, Corich V, Squartini A, Ninke K, Philip-Hollingsworth S, Orgambide G, De Bruijn F, Stoltzfus J, Buckley D, Schmidt TM, Mateos PF, Ladha JK, Dazzo FB (1997) Natural endophytic association between *Rhizobium leguminosarum* bv. trifolii and rice roots and assessment of its potential to promote rice growth. Plant Soil 194:99–114

Yanni YG, Rizk RY, Abd El-Fattah FK, Squartini A, Corich V, Giacomini A, De Bruijn F, Rademaker J, Maya-Flores J, Ostrom P (2001) The beneficial plant growth-promoting association of *Rhizobium leguminosarum* bv. trifolii with rice roots. Aust J Plant Physiol 28:845–870

Zézé A, Mutch LA, Young PW (2001) Direct amplification of *nodD* from community DNA reveals the genetic diversity of *Rhizobium leguminosarum* in soil. Environ Microbiol 3:363–370